Positive selection and relaxed purifying selection contribute to rapid evolution of male-biased genes in a dioecious flowering plant

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Abstract

Sex-biased genes offer insights into the evolution of sexual dimorphism. Sex-biased genes, especially those with male bias, show elevated evolutionary rates of protein sequences driven by positive selection and relaxed purifying selection in animals. Although rapid sequence evolution of sex-biased genes and evolutionary forces have been investigated in animals and brown algae, less is known about evolutionary forces in dioecious angiosperms. In this study, we separately compared the expression of sex-biased genes between female and male floral buds and between female and male flowers at anthesis in dioecious *Trichosanthes pilosa* (Cucurbitaceae). In floral buds, sex-biased gene expression was pervasive, and had significantly different roles in sexual dimorphism such as physiology. We observed higher rates of sequence evolution for male-biased genes in floral buds compared to female-biased and unbiased genes. Male-biased genes under positive selection were mainly associated with functions to abiotic stress and immune responses, suggesting that high evolutionary rates are driven by adaptive evolution. Additionally, relaxed purifying selection may contribute to accelerated evolution in male-biased genes generated by gene duplication. Our findings, for the first time in angiosperms, suggest evident rapid evolution of male-biased genes, advance our understanding of the patterns and forces driving the evolution of sexual dimorphism in dioecious plants.

Key words: *Trichosanthes pilosa*, positive selection, relaxed purifying selection, sexual dimorphism, sex-biased genes, floral development stages

Introduction

Sexual dimorphism presents different morphological, ecological and physiological traits in gonochoristic animals and dioecious plants, despite male and female individuals sharing the same genome except for sex chromosomes (or sex-determining loci) (Mank, 2009; Barrett and Hough, 2013). Such sexual dimorphisms usually arise from differential expression of genes in both sexes, i.e., sex-biased genes (including sex-specific genes exclusively in one sex) that are located on autosomal chromosomes and sex chromosomes/or sex-determining regions (Ellegren and Parsch, 2007; Parsch and Ellegren, 2013; Charlesworth, 2018b). Recently, some studies have begun to explore the strength and impact of evolutionary forces that shape different sexually dimorphic traits through sex-biased gene expression (Mank, 2009; Rowe et al., 2018; Naqvi et al., 2019; Tosto et al., 2023). Previous studies revealed that sex-biased gene expressions were associated with the evolution of sexual dimorphisms in many animals, although the extent to which exhibits great variation among taxa, tissues, and development stages (Mank, 2017; Hsu et al., 2020; Khodursky et al., 2020; Lichilin et al., 2021; Toubiana et al., 2021; Djordjevic et al., 2022; Yue et al., 2023). Unlike most animals, the vast majority (~90%) of flowering plants (angiosperms) are hermaphroditic, while only a small fraction (~5%) are dioecious in which individuals have exclusively male or female reproductive organs (Renner, 2014). Most dioecious plants possess homomorphic sex-chromosomes that are roughly similar in size when viewed by light microscopy.

Furthermore, sexual dimorphism in dioecious plants is less common and less conspicuous than that in most animals (Barrett and Hough, 2013). Hence, the study of sex-biased gene expression is of great interest to plant evolutionary biologists, as it is necessary to understand the evolution of sexual dimorphism in dioecious plants (Moore and Pannell, 2011).

A common pattern that has emerged from previous studies is that sex-biased genes, particularly male-biased genes, tend to evolve rapidly in protein sequence (the ratio of non-synonymous to synonymous substitutions, d_N/d_S) compared to unbiased genes (Ellegren and Parsch, 2007; Grath and Parsch, 2016). The rapid evolution of male-biased genes was first observed in *Drosophila melanogaster* (Zhang et al., 2004; Zhang and Parsch, 2005) and has been supported by recent investigations in a wider range of animals (Pröschel et al., 2006; Mank et al., 2007; Mank, 2017; Papa et al., 2017; Catalan et al., 2018; Toubiana et al., 2021). In recent years, there have been growing studies on the expression dynamics and molecular evolutionary rates of sex-biased genes in flowering plants, including hermaphroditic *Arabidopsis thaliana* (Gossmann et al., 2014; 2016), *Solanum* (Moyle et al., 2021), and dioecious *Silene latifolia* (Zemp et al., 2016), *Salix viminalis* (Darolti et al., 2018), *Mercurialis annua* (Cossard et al., 2019), *Populus balsamifera* (Sanderson et al., 2019), and *Leucadendron* (Scharmann et al., 2021). However, despite such advances, the molecular evolution pattern of sex-biased genes in plants remains inconsistent among the studied plant species (Muyle, 2019; Veltsos, 2019). In dioecious plants such as *Mercurialis annua* and *Leucadendron*, Cossard et al., (2019) and Scharmann et al.,

(2021) found no significant differences in evolutionary rates of proteins among female-biased, male-biased and unbiased genes detected between male and female plants leaf tissues, although the expression of sex-biased genes was highly different from unbiased genes in leaves. Similar patterns have also been reported in dioecious *Populus balsamifera*, where evolutionary rates of male-biased, female-biased and unbiased genes did not differ in reproductive tissues (Sanderson et al., 2019). However, in the dioecious *Salix viminalis*, male-biased genes have significantly lower evolutionary rates of proteins than female-biased and unbiased genes in catkin tissues (Darolti et al., 2018). To our knowledge, only above mentioned five studies have investigated the expression differences and protein evolutionary rates of sex-biased genes in dioecious angiosperms. Moreover, these studies only compared gene expression in vegetative versus vegetative tissues and vegetative versus reproductive tissues, limiting our understanding of sexual selection at different floral development stages. Therefore, more studies and taxa are needed to explore the common patterns of sequence evolution in sex-biased genes, with more focus on comparing sex-biased gene expression in reproductive versus reproductive tissues, e.g., different floral development stages in dioecious angiosperms.

Evolutionary analyses indicate that different driving forces impact the rate of sequence evolution of sex-biased genes. These forces include positive selection, which promotes the spread and adaptive fixation of beneficial alleles; sexual selection, which results from male-male competition or female choice; and relaxed purifying selection, which reduces the removal of deleterious mutations (Grath and Parsch,

2016; Mank, 2017; Dapper and Wade, 2020). For example, in animal systems, particularly in *Drosophia*, the elevated sequence divergence rates of male-biased genes have often been interpreted as the signature of adaptive evolution, suggesting that sexual selection is the primary evolutionary force (Pröschel et al., 2006; Assis et al., 2012). In brown algae, female-biased and/or male-biased genes exhibiting higher evolutionary rates than unbiased genes suggest that rapid evolution is partly driven by adaptive evolution or sexual selection (Lipinska et al., 2015; Cossard et al., 2022; Hatchett et al., 2023). However, studies in plants have rarely reported elevated rates of sex-biased genes.

An alternative explanation for the rapid evolution of sex-biased genes is a relaxation of purifying selection due to reduced constraints (Lahti et al., 2009; Dapper and Wade, 2020). In the model plant *Arabidopsis thaliana*, pollen genes were found to be evolving faster than sporophyte-specific genes due to relaxed purifying selection associated with the transition from outcrossing to selfing (Harrison et al., 2019). These trends were recently confirmed in *Arabis alpina*, which exhibits mating system variation across its distribution, suggesting that the efficacy of purifying selection on male gametophyte-expressed genes was significantly weaker in inbred populations (Gutierrez-Valencia et al., 2022). Together, these findings in plants reinforce the idea that both adaptive (e.g., positive selection, sexual selection) and non-adaptive (e.g., relaxed selection) evolutionary processes differentially impact the sequence evolution of sex-biased genes. Hence, investigating the potential contribution of selection forces to the emergence of specific evolutionary patterns of sex-biased genes within a focal

species is of great interest.

In the family Cucurbitaceae, there are about 96 genera and 1,000 species, about 50% of species are dioecious, and the others are monoecious (Schaefer and Renner, 2011). Phylogenetic analyses of Cucurbitaceae suggest that dioecy is the ancestral state of the family, but transitions frequently to monoecy (Zhang et al., 2006). *Trichosanthes pilosa* (synonym: *T. ovigera*, 2n = 22, Cucurbitaceae) is mainly distributed from Southwest and Southeast China to Japan, extending to Southeast Asia, New Guinea and Western Australia. It was suggested to have originated in the late Miocene (ca. 8-6 million-year ago) (de Boer et al., 2012; 2015; Guo et al., 2020). *Trichosanthes pilosa* is a perennial, night-flowering, insect-pollinated dioecious vine that reproduces sexually and possesses a pair of heteromorphic sex chromosomes XX/XY (Ming et al., 2011). It's male and female flowers exhibit strong sexual dimorphism in floral morphological and phenological traits, such as racemose versus solitary (Fig. 1), early-flowering versus late-flowering, and caducous versus long-lived (Wu et al., 2011).

To understand the evolution of sex-biased genes in dioecious *T. pilosa*, we collected floral buds and flowers at anthesis from male and female individuals and characterized their expression profiles using Illumina RNA sequencing. Our primary objectives are to 1) compare expression divergence between males and females at two floral development stages; 2) explore whether there are differences in the evolutionary rates of proteins among female-biased, male-biased and unbiased genes; and if so, 3) determine the main selective forces that contribute to the differentiation of sequence

evolution rates among gene categories.

Results

Transcriptome sequencing, *de novo* assembly and annotation

Using whole transcriptome shotgun sequencing, we sequenced 12 floral buds and flowers at anthesis from three females and three males of dioecious *T. pilosa* and generated a total of nearly 276 million clean reads (Table S1). Due to the absence of a reference genome, we performed *de novo* assembly of transcripts from all the clean reads, followed by clustering and filtering analysis, resulting in 59,051 unigenes (Fig. S1A). To evaluate the quality of the assembled unigenes, we used BUSCO assessments based on embryophyta_odb10 database, which showed the completeness of the reference transcriptome at 89.7% (Table S2). We then annotated them against protein databases including NR, KEGG, Swissport, PFAM, and GO using BLASTP and nucleotide database NT using BLASTN (Table S2). The e-value distribution of the best hits in NR database suggested that 47,241 unigenes (80%) had strong homology, with an e-value smaller than 1.0e-15 (Fig. S1B). The majority of unigenes were annotated by homologs in species of Cucurbitaceae, such as *Momordica charantia* (16.3%), *Cucumis melo* (11.9%), *Cucurbita pepo* (11.9%), *Cucurbita moschata* (11.5%) and *Cucurbita maxima* (10.1%) (Fig. S1C). Overall, our assessment suggested that we have generated high-quality reference transcriptomes.

173 **Expression characteristics of sex-biased genes**

174 We mapped the RNA-Seq reads of floral buds and flowers at anthesis onto the
175 reference transcriptome in dioecious *T. pilosa*, which resulted in approximately 75%
176 read mappings per sample (Table S3). In floral buds, we identified 5,096 (9.50%)
177 female-biased genes and 4,214 (7.86%) male-biased genes (Fig. 2A). In contrast, only
178 380 (0.70%) female-biased genes and 233 (0.43%) male-biased genes were detected
179 in flowers at anthesis (Fig. 2B). Using hierarchical clustering analysis, we evaluated
180 different levels of gene expression across sexes and tissues (Fig. 2C), revealing that
181 female floral buds maybe tend to masculinize, and vice versa in the early stages of
182 floral development. Furthermore, we observed that the number of sex-biased genes in
183 floral buds was approximately 15 times higher than in flowers at anthesis, indicating
184 that sex-biased genes associated with meiotic processes, sex differentiation and
185 sexually dimorphic traits are predominantly expressed in floral buds. We also
186 analyzed sex-specific genes that were exclusively expressed in floral buds and flowers
187 at anthesis of one sex. In floral buds, we found 253 out of 5,096 (4.96%)
188 female-specific genes and 465 out of 4,214 (11.03%) male-specific genes. However,
189 in flowers at anthesis, we only identified 26 out of 380 (6.84%) female-specific genes
190 and 52 out of 233 (22.32%) male-specific genes. Taken together, sex bias is more
191 prevalent in floral buds than in flowers at anthesis.

192 **Tissue-biased/stage-biased gene expression**

193 We compared the expression levels of transcripts in floral buds and flowers at

194 anthesis within each sex to identify genes with tissue-biased expression. In male
195 plants, the number of tissue-biased genes in flowers at anthesis was 1,040 higher than
196 that in floral buds (Fig. 3A and 3B). However, in female plants, the number of
197 tissue-biased genes in flowers at anthesis was only 536 more than that in floral buds
198 (Fig. 3C and 3D). Our results indicated that males had a higher tissue-bias relative to
199 females. We also identified sex-biased genes that were expressed in both types of
200 tissues by comparing tissue-biased genes with male-biased and female-biased genes,
201 respectively. Few female-biased genes overlapped with tissue-biased genes,
202 accounting for only 85 out of 5,096 (1.67%) in floral buds (Fig. 3C) and 5 out of 380
203 (1.32%) in flowers at anthesis (Fig. 3D). However, a significant proportion of
204 male-biased genes overlapped with tissue-biased genes, with 1,010 out of 4,214
205 (23.97%) in floral buds (Fig. 3A) and 145 out of 233 (62.23%) in flowers at anthesis
206 (Fig. 3B).

207 **Elevated protein evolutionary rates of male-biased genes in floral buds**

208 In four species with phylogenetic relationships (((*T. anguina*, *T. pilosa*), *T.*
209 *kirilowii*), *Luffa cylindrica*), including dioecious *T. pilosa*, dioecious *T. kirilowii*,
210 monoecious *T. anguina* in *Trichosanthes*, together with monoecious *Luffa cylindrica*,
211 we identified 1,145 female-biased, 343 male-biased, and 2,378 unbiased one-to-one
212 orthologous groups (OGs) from floral buds. Additionally, we detected 45
213 female-biased, 13 male-biased, and 3,782 unbiased one-to-one OGs from mature
214 flowers in all four species. To quantify the rates of protein sequences, we separately

215 calculated ω values for each sex-biased and unbiased orthologous gene using
216 'two-ratio' and 'free-ratio' branch models in juvenile and mature flowers (Figs. 4 and
217 S2).

218 The two-ratio branch model, where the foreground (dioecious branches) has a
219 different ω value relative to the background (all other branches), is better supported
220 than the fixed-ratio branch model, where the same ω value was in all branches. In the
221 results of 'two-ratio' branch model, the median of ω values in female-biased,
222 male-biased and unbiased genes were 0.227, 0.257 and 0.230 in floral buds,
223 respectively (Fig. 4A and Table S4). We observed that male-biased genes had a 13.22%
224 and 11.74% higher median than female-biased and unbiased genes in floral buds,
225 respectively. The difference in the distribution of ω values between female-biased
226 versus male-biased genes (P = 0.0021) and male-biased versus unbiased genes (P =
227 0.0051) was statistically significant in Wilcoxon rank sum tests. However, we did not
228 find a significant difference in ω values between female-biased and unbiased genes in
229 floral buds (Wilcoxon rank sum test, P = 0.4618). In flowers at anthesis, the median
230 of ω values for female-biased, male-biased, and unbiased genes were 0.269, 0.177 and
231 0.231, respectively (Fig. 4B and Table S4). However, we found that there was no
232 statistically significant difference in the distribution of ω values using Wilcoxon rank
233 sum tests for female-biased versus male-biased genes (P = 0.0556), female-biased
234 versus unbiased genes (P = 0.0796), and male-biased versus unbiased genes (P =
235 0.3296).

236 In free-ratios model, ω values are free to vary in each branch compared to

237 fixed-ratio branch model and two-ratio branch model. The 'free-ratio' branch model
238 yielded interesting results. In floral buds, the median ω values for female-biased,
239 male-biased, and unbiased genes were 0.222, 0.265 and 0.226, respectively (Fig. 4C
240 and Table S5). Male-biased genes had a significantly higher median relative to
241 female-biased genes (19.37% higher, Wilcoxon rank sum test, $P = 0.0009$) and
242 unbiased genes (17.26% higher, Wilcoxon rank sum test, $P = 0.0004$) in floral buds.
243 However, there was no significant difference in ω values between female-biased and
244 unbiased genes (Wilcoxon rank sum test, $P = 0.9862$). In flowers at anthesis, the
245 median ω values for female-biased, male-biased, and unbiased genes were 0.300,
246 0.148 and 0.227, respectively (Fig. 4D and Table S5). Female-biased and unbiased
247 genes had significantly higher ω values than male-biased (Wilcoxon rank sum test, P
248 $= 0.0101$, $P = 0.0146$, respectively). However, there was no significant difference in ω
249 values between female-biased and unbiased genes (Wilcoxon rank sum test, $P =$
250 0.2887). Since the number of male-biased genes and evolutionary rates of male-biased
251 genes in flowers at anthesis are lower than those in floral buds, we decided to focus
252 on the latter in subsequent analyses.

253 **Evidence of positive selection and relaxed selection for male-biased genes in**
254 **floral buds**

255 After comparing the A model and null model (see Methods Section), we
256 discovered that 39 out of 343 OGs (11.34%) in male-biased genes of floral buds
257 exhibited strong evidence of having certain sites that evolved under positive selection

258 based on foreground 2b ω value, likelihood ratio tests (LRTs, $P < 0.05$) and Bayes
259 empirical Bayes (BEB) value (Fig. 5 and Table S6). As an additional approach, we
260 utilized the aBSREL and BUSTED methods that were implemented in HyPhy v.2.5
261 software, and detected significant evidence of positive selection. According to our
262 findings, 84 out of 343 OGs (24.49%) were identified to be under episodic positive
263 selection in male-biased genes of floral buds with a site proportion of 0.17%–26.44%
264 based on aBSREL (Table S7). In addition, 69 out of 343 OGs (20.01%) exhibited
265 significant signs of positive selection with the site proportion of 0.28%–32.65% in
266 male-biased genes of floral buds according to BUSTED (Table S8). Among these, a
267 total of 32 OGs (9.30%) were identified through our tests using CodeML, aBSREL
268 and BUSTED (Fig. 5).

269 Relaxed selection may occur when the efficiency of natural selection (e.g., the
270 reduction of the strength of purifying selection) is dramatically reduced or eliminated
271 at both gene-wide and genome-wide levels, leading to accumulations of deleterious
272 mutations relative to positive selection (Lahti et al., 2009; Dapper and Wade, 2020).
273 This has been proposed as an explanation for the rapid evolution of sex-biased genes
274 (Lahti et al., 2009; Mank, 2017). Using the RELAX model, we conducted an analysis
275 and found that 18 out of 343 OGs (5.23%) showed significant evidence of relaxed
276 selection (K = 0.0184–0.6497) (Tables S9 and S13). Interestingly, we also observed
277 that 61 out of 343 OGs (17.73%) exhibited significant evidence of intensified positive
278 selection (K = 2.3363–50, $\omega_2 \geq 1$) (Fig. 5 and Table S10), which is consistent with the
279 results obtained from CodeML, aBSREL and BUSTED.

280 According to previous studies (Ellegren and Parsch, 2007; Catalan et al., 2018),
281 genes that exhibit sex-biased expression with rapid evolutionary rates tend to display
282 a lower codon bias compared to unbiased genes. In our results, we found that
283 male-biased genes in floral buds had a significantly lower median effective number of
284 codons (ENCs) than both female-biased and unbiased genes (Wilcoxon rank sum test,
285 female-biased vs male-biased genes, $P = 0.0001$ and male-biased vs unbiased genes,
286 $P = 0.0123$). This suggested that male-biased genes in floral buds exhibit stronger
287 codon bias than both female-biased and unbiased genes (Fig. S3). In short, our
288 analyses indicated that rapid evolutionary rates of male-biased genes in floral buds
289 were not associated with a reduction in codon usage bias.
290 To confirm the contributions of positive selection and relaxed selection to rapid
291 rates of male-biased genes in floral buds, we generated three datasets of OGs by
292 excluding different sets of genes. Specifically, we excluded 18 relaxed selective
293 male-biased genes (5.23%), 98 positively selected male-biased genes (28.57%), and
294 112 male-biased genes (32.65%) under positive and relaxed selection from 343 OGs
295 (Fig. S4). We observed that after excluding male-biased genes under relaxed purifying
296 selection, the median (0.264) decreased by 0.34% compared to the median (0.265) of
297 all OGs (Fig. S4A-B). However, after excluding positively selected male-biased genes,
298 the median (0.236) was reduced by 11% (Fig. S4A, C) in the results of 'free-ratio'
299 branch model. This pattern was consistent with the results of 'two-ratio' branch model
300 as well (Fig. S4E-G). Additionally, we analyzed female-biased and unbiased genes
301 that underwent positive and relaxed selection in floral buds (Tables S6-S10). We

302 identified 216 (18.86%) positively selected (Fig. S5A), and 69 (6.03%) relaxed
303 selective female-biased genes from 1,145 OGs (Fig. S5B), respectively. Similarly, we
304 found 436 (18.33%) positively selected (Fig. S6A), and 43 (1.81%) unbiased genes
305 under relaxed selection from 2,378 OGs (Fig. S6B), respectively. Notably,
306 male-biased genes have a higher proportion (10%) of positively selected genes
307 compared to female-biased and unbiased genes. However, relaxed selective
308 male-biased genes have a higher proportion (3.24%) than unbiased genes, but about
309 0.8% lower than that of female-biased genes. In summary, our analyses suggested that
310 positive selection and relaxed selection likely drove the rapid evolutionary rates of
311 male-biased genes compared to female-biased and unbiased genes in floral buds, but
312 positive selection had an even greater impact.

313 **Functional analysis of sex-biased genes in floral buds**

314 We conducted KEGG pathway enrichment analysis on sex-biased genes in floral
315 buds. Our results showed that 699 genes were female-biased and 358 genes were
316 male-biased, with significant enrichment ($P < 0.05$) in 26 and 24 KEGG pathways,
317 respectively (Table S11). In the floral bud stage, we observed that female-biased
318 genes were mainly enriched in metabolic and signaling pathways, such as ribosome,
319 Fatty acid elongation, photosynthesis and plant hormone signal transduction (Fig. S7A
320 and Table S11). On the other hand, male-biased genes were significantly enriched in
321 metabolic and signaling pathways, including inositol phosphate metabolism, starch
322 and sucrose metabolism, regulation of autophagy, plant hormone signal transduction,

323 and Toll-like receptor signaling pathway (Fig. S7B and Table S11).

324 We have also found that certain male-biased genes, which are evolving under
325 positive selection and relaxed selection (Table S12 and S13), were related to abiotic
326 stress and immune responses. For instance, mitogen-activated protein kinase kinase
327 kinase 18 (MAPKKK18) (Zhang and Zhang, 2022), zinc finger CCCH
328 domain-containing protein 20 (C3H20/TZF2) (Bogamuwa and Jang, 2014), and heat
329 stress transcription factor B-3 (HSFB3) (Scharf et al., 2012) have been linked to stress.
330 Additionally, ten male-biased genes with rapid evolutionary rates were associated
331 with anther and pollen development. These genes include LRR receptor-like
332 serine/threonine protein kinase (LRR-RLK) (Cui et al., 2022), pollen receptor-like
333 kinase 3 (*PRK3*) (Muschietti and Wengier, 2018), autophagy-related protein 18f
334 (*ATG18f*) (Zhou et al., 2015; Li et al., 2020), and plant homeodomain (PHD) finger
335 protein 3 (*MALE STERILITY 3*) (Hou et al., 2022) in floral buds of male plants.

336 **Discussion**

337 The Cucurbitaceae family, where half of the species are monoecious and half are
338 dioecious, is an excellent model for studying the evolution of sexual systems of
339 angiosperms, including sex-determination mechanism and sexual dimorphism
340 (Schaefer and Renner, 2010; Boualem et al., 2015; Ma and Pannell, 2016). In this
341 study, we aimed to investigate whether sex-biased genes exhibited evidence of rapid
342 evolutionary rates of protein sequences and identify the evolutionary forces
343 responsible for the observed patterns in the dioecious *Trichosanthes pilosa*. We

compared the expression profiles of sex-biased genes between sexes and two tissue types and examined the signatures of rapid sequence evolution for sex-biased genes, as well as the contributions of potential evolutionary forces.

Sex-biased expression in floral buds

Numerous studies have shown that in dioecious plants, male-biased genes tend to outnumber female-biased genes, consistent with the patterns in most animals (Zhang et al., 2007; Djordjevic et al., 2022). For instance, insect-pollinated dioecious plants such as *Asparagus officinalis* (Harkess et al., 2015) and *Silene latifolia* (Zemp et al., 2016), exhibit a higher proportion of male-biased genes, whereas wind-pollinated dioecious plants like *Populus balsamifera* (Sanderson et al., 2019) has twice as many female-biased genes than male-biased genes. The differences in these studies could be partly attributed to the impact of sexual selection on secondary sexual traits in insect-pollinated dioecious plants, as opposed to wind-pollinated ones (Delph and Herlihy, 2012; Muyle, 2019; Sanderson et al., 2019). In contrast to the above studies, our findings revealed that the number of female-biased genes in floral buds of the night-flowering, insect-pollinated dioecious plant *Trichosanthes pilosa* exceeded that of male-biased genes by 882 (~21%). This excess of female-biased expression could be due to lower energy consumption needs and reduced chemical defense capability against insect herbivores in short-lived male flowers (Sanderson et al., 2019). Indeed, functional enrichment analysis in chemical pathways such as terpenoid backbone and diterpenoid biosynthesis indicated that relative to male floral buds, female floral buds

365 had more expressed genes that were equipped to defend against herbivorous insects
366 and pathogens, except for growth and development (Vaughan et al., 2013; Ren et al.,
367 2022) (Fig. S7A and Table S11). Additionally, our enrichment analysis showed that
368 the photosynthesis, porphyrin and chlorophyll metabolism pathways were more active
369 in female floral buds compared to male floral buds (Fig. S7A and Table S11), enabling
370 them to acquire more resources such as carbon for fruit and seed production
371 (Delph,1999).
372 We identified functional enrichments in Toll-like receptor signaling, NF-kappa B
373 signaling, and inositol phosphate metabolism pathways in male floral buds (Fig. S7B,
374 Table S11). We also found that male-biased genes with high evolutionary rates in male
375 floral buds were associated with functions to abiotic stresses and immune responses
376 (Tables S12 and S13), which suggest that male floral buds through rapidly evolving
377 genes are adapted to mountain climate and the environment in Southwest China
378 relative to female floral buds through high gene expression. In addition, the
379 enrichment in regulation of autophagy pathways could be associated with gamete
380 development and the senescence of male floral buds (Table S14) (Liu and Bassham,
381 2012; Li et al., 2020; Zhou et al., 2021). In fact, it was observed that male flowers
382 senesced faster (Wu et al., 2011). We also found that homologous genes of two
383 male-biased genes in floral buds (Table S14) that control the raceme inflorescence
384 development (Teo et al., 2014) were highly expressed compared to female floral buds.
385 Taken together, these results indicate that expression changes in sex-biased genes,
386 rather than sex-specific genes play different roles in sexual dimorphic traits in

physiology and morphology (Dawson and Geber, 1999).

Rapid Evolution of male-biased genes in floral buds

It has been observed that, in most animals, sex-biased genes, particularly those biased towards males, often exhibit more rapid evolutionary rates than unbiased genes (Parsch and Ellegren, 2013; Grath and Parsch, 2016; Mank, 2017; Toubiana et al., 2021). However, in dioecious angiosperms, no evidence of rapid evolution in sex-biased genes relative to unbiased genes has been found (Zemp et al., 2016; Darolti et al., 2018; Cossard et al., 2019; Sanderson et al., 2019; Scharmann et al., 2021). In contrast, our findings indicated that male-biased genes experience higher evolutionary rates than both female-biased and unbiased genes in floral buds of dioecious *T. pilosa.* We proposed that positive selection and relaxed purifying selection may be responsible for the rapid sequence evolution of male-biased genes.

After analyzing the data, we found that around 28.57% (98 genes) of male-biased genes have undergone positive selection. Additionally, we observed that the proportion of male-biased genes under positive selection was about 10% higher than that of female-biased and unbiased genes. Furthermore, we discovered that some male-biased genes under positive selection were linked to abiotic stress and immune responses (Table S12). Our findings are consistent with studies on *Drosophila* and *Ectocarpus* (Zhang and Parsch, 2005; Lipinska et al., 2015), suggesting that adaptive evolution is one of the important driving forces for rapid evolutionary rates. Notably, we identified several male-biased genes under positive selection that are functionally

408 related to early flowering (*phyB*) (Stephenson and Bertin, 1983; Forrest, 2014; Hajdu
409 et al., 2015) and pollen development (Skogsmyr and Lankinen, 2002; Williams and
410 Reese, 2019) (Tables S12- S14). These findings indicate that a small fraction of
411 male-biased genes may experience adaptive evolution due to sexual selection, driven
412 by male-male competition.

413 Alternatively, relaxed constraints could contribute to the rapid evolutionary rates
414 of sex-biased genes through three key characteristics (Dapper and Wade, 2020; Tosto
415 et al., 2023). First, sex-biased genes are often expressed solely in reproductive tissues
416 of one sex (e.g., sex-specific genes), particularly in the haploid phase (Immler, 2019;
417 Beaudry et al., 2020). Sex-specific selection (e.g., relaxed purifying selection) acting
418 on sex-specific genes could decrease the elimination of deleterious mutations (Mank,
419 2017), such as pollen-specific (Harrison et al., 2019) or testes-specific genes
420 (Gershoni and Pietrokovski, 2014). However, our results observed male-biased genes
421 but not male-specific genes that underwent relaxed purifying selection. Second,
422 sex-biased genes are often expressed in few tissues (tissue-biased genes) (Meisel,
423 2011; Tosto et al., 2023), resulting in these genes rapidly evolving under positive
424 selection or under relaxed purifying selection due to low pleiotropic constraints
425 (Congrains et al., 2018; Whittle et al., 2021; Tosto et al., 2023). In our results, 343
426 male-biased genes with faster evolutionary rates relative to female-biased and
427 unbiased genes overlapped with tissue-biased genes in floral buds (27 out of 343,
428 7.87%) (Fig. S8A). Furthermore, 27 out of 343 male-biased genes (that is,
429 tissue-biased genes) in floral buds overlapped with nine out of 98 (9.18%)

430 male-biased genes under positive selection (Fig. S8B), and one out of 18 (5.56%)
431 male-biased genes under relaxed purifying selection (Fig. S8C). So, we obtained ten
432 rapidly evolving tissue-biased genes which were also male-biased in male flower buds,
433 suggesting that elevated evolutionary rates may partly be linked to low pleiotropy,
434 consistent with male-biased genes in *Fucus* (Hatchett et al., 2023). Finally, gene
435 duplication has long been thought to promote functional divergences and phenotypic
436 novelties by relaxing the constraints of purifying selection on the duplicated gene
437 copy early in its history (Lynch and Conery, 2000; Lynch and Katju, 2004; Lahti et al.,
438 2009). For instance, the progesterone receptor gene family in the human lineage
439 (Marinić and Lynch, 2020) and the CYP98A9 clade in Brassicales (Liu et al., 2016)
440 have demonstrated rapid evolution and divergent function due to relaxed purifying
441 selection. In our results, we identified 18 out of 343 (5.25%) male-biased genes that
442 underwent relaxed purifying selection using RELAX model (Table S13). Interestingly,
443 the vast majority of genes under relaxed selection were members of different gene
444 families generated by gene duplication (including whole-genome duplication), such as
445 LOB domain-containing protein 18 (*LBD18*) (Zhang et al., 2020), WRKY
446 transcription factor 72 (*WRKY72*) (Chen et al., 2017), and pollen receptor-like kinase
447 3 (*PRK3*) (Muschietti and Wengier, 2018).
448 Additionally, reducing codon usage bias could theoretically accelerate
449 evolutionary rates of sex-biased genes by decreasing synonymous substitution rates.
450 However, our results did not support this idea due to stronger codon usage bias in
451 male-biased genes. Codon usage bias is influenced by many factors, such as levels of

452 gene expression. Highly expressed genes have a stronger codon usage bias and could
453 be encoded by optimal codons for more efficient translation (Frumkin et al., 2018;
454 Parvathy et al., 2022), consistent with high levels of gene expression in males (that is,
455 male-biased genes) in floral buds. Additionally, stronger codon usage bias may be
456 related to higher synonymous substitution rates (Parvathy et al., 2022). Indeed,
457 male-biased genes had significantly higher median d_S values than female-biased and
458 unbiased genes, both in the 'free-ratio' analysis (Fig. S9A, female-biased vs
459 male-biased genes, P = 6.444e-12 and male-biased vs unbiased genes, P = 4.564e-13)
460 and 'two-ratio' branch model (Fig. S9B, female-biased versus male-biased genes, P =
461 2.2e-16 and male-biased versus unbiased genes, P = 9.421e-08, respectively). Hence,
462 rapid evolution of male-biased genes in floral buds was not associated with codon
463 usage bias in our results.

464 Several species have been observed to exhibit rapid evolutionary rates of
465 sequences on sex chromosomes compared to autosomes, which has been related to the
466 evolutionary theories of fast-X or fast-Z (Hough et al., 2014; Wright et al., 2015;
467 Charlesworth et al., 2018a). Furthermore, the quantification of gene expression by
468 bulk RNA-seq technology, relative to single-cell transcriptome analysis, has been
469 shown to potentially obfuscate true signals in the evolution of sex-biased gene
470 expression in complex aggregations of diverse cell types (Darolti and Mank, 2023;
471 Tosto et al., 2023). However, investigation of these interesting issues related to
472 sex-biased gene evolution in *T. pilosa* can only be conducted when whole genome
473 sequences become available in the near future.

474 **Methods**

475 **Plant materials and RNA isolation**

476 Floral buds (≤ 3 mm) and flowers at anthesis were sampled from three female
477 and three male plants (Fig. 1) from the mountainous regions of Anning (Qinglong
478 Gorge), Yunnan Province in Southwest China. Floral buds from female and male
479 plants were named F1 and M1, respectively. Similarly, flowers at anthesis from
480 female and male plants, there were named F2 and M2, respectively (Table S1). To
481 exclude possible bacterial contamination, all tissues were sterilized with 75% alcohol
482 and immediately rinsed with purified water. All samples were then snap-frozen in
483 liquid nitrogen, and stored at -80˚C. Total RNA was extracted from each sample using
484 TRIzol reagent (Life Technologies, CA, USA) according to the manufacturer's
485 instructions. The quantification and qualification of RNA were assessed by the RNA
486 Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA,
487 USA).

488 **Illumina sequencing, *de novo* assembly and annotation**

489 To construct the library, approximately 2 μg of total RNA was used with the
490 Illumina NEBNext UltraTM RNA Library Prep Kit. RNA sequencing was performed
491 on the Illumina NovaSeq 6000, generating 150 bp paired-end reads. The resulting
492 clean reads were obtained by removing adapters, reads containing N bases and
493 low-quality reads using Trimmomatic v.0.39 (Bolger et al., 2014). These reads were

494 deposited in the NCBI database (PRJNA899312).

495 *De novo* assembly for clean reads from all samples was performed using Trinity
496 v.2.10.0 (Haas et al., 2013) with min_kmer_cov: 3 and all other default parameters. To
497 eliminate contamination, all transcripts of *de novo* assembly were compared to
498 bacterial genomes downloaded from NCBI databases using BLASTN with e-value of
499 1.0e-05 in blast+ 2.12.0 software. We used Corset v.4.6 (Davidson and Oshlack, 2014)
500 to obtain high quality, non-redundant consensus transcripts (unigenes). TransDecoder
501 v.5.5.0 was run with -m 100 parameters, namely at least 100 amino acids, to predict
502 the coding DNA and protein sequences (Haas et al., 2013).

503 To evaluate the accuracy and completeness of reference transcriptomes, we
504 performed gene function annotations based on the following databases, using BLAST
505 with a cutoff e-value of 1.0e-05: NR, NT and Swissport (Shiryev et al., 2007). We
506 mapped the unigenes to Pfam database using InterProScan v.5.41 (Jones et al., 2014),
507 to the GO database using Blast2GO (Conesa et al., 2005), and to the KEGG database
508 using KEGG automatic annotation server (Moriya et al., 2007). Additionally, we
509 estimated the completeness of reference transcriptomes using BUSCO v.5.4.5 based
510 on embryophyta_odb10 database (Seppey et al., 2019).

511 **Detection of sex-biased genes**

512 Clean reads were mapped onto all unigenes using Bowtie2 (Langmead and
513 Salzberg, 2012). Read counts were normalized to FPKM (Fragments Per Kilobase
514 Million) value for each unigene using RSEM (Li and Dewey, 2011) in different male

515 and female samples. Genes with zero read counts (i.e., no expression) in both two
516 sexes and tissues were excluded. Differential expression analysis between sexes and
517 tissue types was performed using DESeq2 R package (Love et al., 2014). Unigenes
518 with an FDR-adjusted $P < 0.05$ and an absolute value of $\log_2$ ratio ≥ 1 identified by
519 DESeq2 were considered as sex-biased genes. To perform KEGG functional
520 enrichment, we used all KEGG annotation terms for all genes as the background and
521 performed the analyses using KOBAS v.2.0.12 (Mao et al., 2005).

522 **Evolutionary rate analyses**

523 To quantify the evolutionary rates of sex-biased genes, we download published
524 genome datasets for monoecious *Trichosanthes anguina* (Ma et al., 2020) and
525 monoecious *Luffa cylindrica* which has a closer phylogenetic relationship with
526 *Trichosanthes* (de Boer et al., 2012; Wu et al., 2020) from CuGenDB database (Zheng
527 et al., 2019). Additionally, we also download published RNA sequencing reads of
528 floral buds and flowers from CNCB (Accession CRA002313) and NCBI databases
529 (Accession SRR5259239) for dioecious plant *Trichosanthes kirilowii* (Hu et al., 2020),
530 and *de novo* assembled by previously described methods.

531 We identified one-to-one OGs using OrthoFinder v.2.3.3 with default parameters
532 from *T. anguina*, *T. pilosa*, *T. kirilowii*, and *Luffa cylindrica* (Emms and Kelly, 2019).
533 Then, we employed TranslatorX with -c 1 -p M -g -b5 n parameters (i.e., the multiple
534 alignment and the trimming using Muscle and GBlocks, respectively), translated
535 nucleotide sequences and back-translated amino acid alignments into nucleotide

536 alignments to ensure codon-to-codon alignment (Abascal et al., 2010). The remaining
537 gapless alignments (≥100 bp in length) were retained.
538 To investigate the evolutionary rates of coding sequences, we estimated
539 nonsynonymous substitution (d_N), synonymous substitution (d_S) rates, as well as
540 protein substitution rates (d_N/d_S, ω), using two branch models from CodeML package
541 in PAML v.4.9h with the F3x4 codon frequencies (CodonFreq = 2) (Yang, 2007).
542 According to the phylogenetic relationships of *Trichosanthes* (de Boer et al., 2012;
543 Guo et al., 2020), we set up tree structures ((*T. anguina*, *T. pilosa*), *T. kirilowii*, *L.*
544 *cylindrica*) in the control file of CodeML. First, we employed a 'two-ratio' branch
545 model (model = 2, Nssites = 0) that assumes the foreground (two dioecious species)
546 has a different ω value from the background (two monoecious species) to estimate and
547 compare the divergences of the foreground. Second, to reduce the potential bias of ω
548 value due to the conflation of two dioecious species, we also implemented a
549 'free-ratio' branch model (model = 1, Nssites = 0), which assumes an independent ω
550 ratio for each branch. Finally, to avoid the effects of saturation substitution, we used
551 separately OGs with $0 < \omega < 2$ and all OGs with $\omega > 0$, plotted the distribution of ω
552 values and compared the median of ω values in female-biased, male-biased and
553 unbiased orthologous genes of floral buds and flowers at anthesis. All comparisons
554 between sex-biased and unbiased genes were tested using Wilcoxon rank sum test in
555 R software.

556 **Estimation of the strength of natural selection**

557 The rapid evolutionary rates of sex-biased genes may be attributed to positive
558 selection, relaxed selection and lower codon usage bias (Catalan et al., 2018; Dapper
559 and Wade, 2020). Therefore, we conducted separate analyses using classical
560 branch-site models, the adaptive branch-site random effects likelihood (aBSREL)
561 model (Smith et al., 2015), the branch-site unrestricted statistical test for episodic
562 diversification (BUSTED) model (Murrell et al., 2015), the RELAX model (Wertheim
563 et al., 2015), and the effective number of codons (ENC) in PAML v.4.9h
564 (Álvarez-Carretero et al., 2023), HyPhy v.2.5 (Pond et al., 2020) and CodonW v.1.4.2
565 (http://codonw.sourceforge.net) to distinguish which evolutionary forces are driving
566 the rapid evolutionary rates of sex-biased genes.

567 To determine if amino acid sites in the foreground, including the *T. pilosa* lineage
568 have undergone positive selection (foreground 2b $\omega > 1$) compared with the
569 background for each OGs, we followed the steps of Zhang et al. (2005), and used
570 branch-site model A (model = 2, Nssite = 2, fix_omega = 0, omega = 1.5) and
571 branch-site model null (model = 2, Nssite = 2, fix_omega = 1, omega = 1). We
572 examined the significance of likelihood ratio tests (LRTs, $P < 0.05$) to identify
573 positively selected sites between model A and model null by comparing LRTs to the
574 Chi-square distribution with two degrees of freedom. We adjusted the LRTs *P* value
575 for multiple comparisons using Benjamini and Hochberg's (FDR) algorithm. When
576 the *P* value was significant, we used Bayes Empirical Bayes (BEB) estimates to

identify sites with a high posterior probability (pp ≥ 0.95) of being under positive selection (Yang et al., 2005).

To detect episodic positive selection at a proportion of sites on the foreground branch, we employed the aBSREL method (Smith et al., 2015) in the HyPhy v.2.5 packages to compare the fully adaptive model ($\omega > 1$) to the null model that allows no positive selection rate classes by LRTs, which is an improved algorithm of branch-site models in PAML. For relatively small datasets, such as those with fewer than 10 taxa, the aBSREL method may not have enough power to detect positive selection. Therefore, we also ran the BUSTED method to identify gene-wide evidence of episodic positive selection at least one site on at least one branch (Murrell et al., 2015). We set *T. pilosa* as the foreground and assessed the statistical significance ($P < 0.05$) using LRTs with the Holm-Bonferroni correction.

To test the relaxation of selective strength, we utilized the RELAX model in the HyPhy v.2.5 software (Wertheim et al., 2015; Schrader et al., 2021). The RELAX model estimates three ω parameters ($\omega_0 \leq \omega_1 \leq 1 \leq \omega_2$), and determines the proportion of sites in the test (foreground) and reference (background) branches using a branch-site model. The first two ω classifications indicate that sites have undergone purifying selection, and the third classification indicates that sites have been under positive selection. Additionally, the model introduces a selection intensity parameter (K value) to compare a null model (K = 1) with an alternative model, thereby assessing the strength of natural selection. When K > 1, it suggests intensified natural selection, when K < 1, indicates relaxed natural selection in the test branch relative to

599 the reference branch. We quantified the statistical confidence of K value ($P < 0.05$)
600 using LRTs and the Holm-Bonferroni correction.

601 To investigate codon usage bias, which refers to the differences in the frequency
602 of occurrence of synonymous codons in coding DNA, we employed CodonW v.1.4.2.
603 This program considers the ENC values from 20 to 61 as a measure of the departure
604 of the genetic codes for a given gene (Wright, 1990), with lower ENC values
605 represent stronger codon usage bias (Hambuch and Parsch, 2005). We performed a
606 Wilcoxon rank sum test to determine if there were deviations in ENC values among
607 female-biased, male-biased, and unbiased genes in floral buds.

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Author contributions

HTL, DZL and LZ conceived the project the study. HTL and LZ designed and performed the experiment. LZ, JH and HTL collected the samples. LZ analyzed the data and wrote the manuscript. HTL, DZL, LZ, and WZ revised the manuscript. All authors approved the final manuscript.

Additional files

Supplementary files

Supplementary file 1. Supplementary Figures S1 to S9.

Supplementary Figure S1. The length distribution of unigenes (A) and the e-value distribution (B) and the species distribution (C) of BLAST hits for each unigene.

Supplementary Figure S2. Boxplot of dN/dS values (including all ω values) of female-biased, male-biased and unbiased genes in floral buds and flowers at

anthesis of *Trichosanthes pilosa*. White dot indicates the median of dN/dS values for sex-biased and unbiased genes. Wilcoxon rank sum tests are used to test for significant differences (***$P < 0.0005$, **$P < 0.005$ and *$P < 0.05$). The distributions of dN/dS values for female-biased, male-biased and unbiased genes in floral buds (A) and flowers at anthesis (B) using 'free-ratio' branch model. The distributions of dN/dS values for female-biased, male-biased and unbiased genes in floral buds (C) and flowers at anthesis (D) using 'two-ratio' branch model.

Supplementary Figure S3. Violin plots of ENCs values of female-biased, male-biased and unbiased genes in floral buds. Significant differences using Wilcoxon rank sum tests are represented by * (***$P < 0.0005$ and *$P < 0.05$).

Supplementary Figure S4. Boxplot of d_N/d_S values of male-biased genes, including all 343 OGs (A and D), excluding 18 OGs under relaxed selection (B and F), excluding 98 OGs under positive selection (C and G), and excluding 112 OGs under both positive and relaxed selection (D and H), and boxplot of d_N/d_S values of all female-biased and unbiased genes in floral buds using 'free-ratio' and 'two-ratio' branch model.

Supplementary Figure S5. Venn and Pie diagrams of female-biased genes under positive selection and relaxed selection in floral buds. Overlaps of female-biased genes were detected to be under positive selection using aBSREL, BUSTED, CodeML and RELAX (A), and to be under relaxed selection using RELAX (B).

Supplementary Figure S6. Venn and Pie diagrams of unbiased genes under positive selection and relaxed selection in floral buds. Overlaps of unbiased genes

661 were identified to be under positive selection using aBSREL, BUSTED, CodeML
662 and RELAX (A), and to be under relaxed selection using RELAX (B).
663 Supplementary Figure S7. Scatterplots of KEGG pathway of sex-biased genes in
664 female (A) and male (B) floral buds of the dioecious *Trichosanthes pilosa*. The y
665 axis represents the pathway name, and the x axis represents the ratio of genes
666 corresponding to the pathways.
667 Supplementary Figure S8. The overlap between male-biased genes with faster
668 evolutionary rates and tissue-biased genes in floral buds. 343 male-biased genes
669 with faster evolutionary rates overlapped (27 out of 343) with tissue-biased genes
670 (A). 27 out of 343 male-biased genes (that is, tissue-biased genes) overlapped
671 with nine out of 98 (B) male-biased genes under positive selection. 27 out of 343
672 male-biased genes overlapped with one out of 18 (C) male-biased genes under
673 relaxed purifying selection.
674 Supplementary Figure S9. Boxplot of d_S values of female-biased, male-biased
675 and unbiased genes in floral buds of dioecious *Trichosanthes pilosa* using
676 'free-ratio' (A) and 'two-ratio' (B) branch model. Significant differences are
677 represented by * in Wilcoxon rank sum tests (***$P < 0.0005$).
678 Supplementary file 2. Supplementary Tables S1 to S14.
679 Supplementary Table S1. Overview of sequencing reads from 12 samples of male
680 and female plants in *Trichosanthes pilosa*.
681 Supplementary Table S2. Numbers of unigenes annotated in public databases.
682 Supplementary Table S3. The mapping rate of reads for each sample in floral

683 buds and flowers at anthesis of *Trichosanthes pilosa*.

684 Supplementary Table S4. d_N, d_S and ω values of each female-biased, male-biased,

685 unbiased orthologous genes of floral buds and flowers at anthesis for each species

686 using 'two-ratio' branch model of CodeML in PAML. Two dioecious species

687 *Trichosanthes pilosa* and *T. kirilowii* represent the foreground and two

688 monoecious species *T. anguina* and *Luffa cylindrica* represent the background.

689 Supplementary Table S5. d_N, d_S and ω values of each female-biased, male-biased,

690 unbiased orthologous genes of floral buds and flowers at anthesis for each species

691 using 'free-ratio' branch model of CodeML in PAML.

692 Supplementary Table S6. Genes under positive selection identified by branch-site

693 model of CodeML in PAML and function in NR, KEGG, Swissport and GO

694 databases for male-biased orthologous genes in floral buds. Two dioecious

695 species *Trichosanthes pilosa* and *T. kirilowii* represent the foreground and two

696 monoecious species, *T. anguina* and *Luffa cylindrica* represent the background.

697 Supplementary Table S7. Genes under episodic positive selection tested by

698 aBSREL model in HyPhy and functions in NR, KEGG, Swissport and GO

699 databases for male-biased orthologous genes in floral buds. The dioecious species

700 *Trichosanthes pilosa* represents the foreground and other species represent the

701 background.

702 Supplementary Table S8. Genes under episodic positive selection found by

703 BUSTED model in HyPhy and functions in NR, KEGG, Swissport and GO

704 databases for male-biased orthologous genes in floral buds. The dioecious species

705 *Trichosanthes pilosa* represents the foreground and other species represent the
706 background.
707 Supplementary Table S9. Genes under relaxed selection detected by RELAX
708 model in HyPhy and functions in NR, KEGG, Swissport and GO databases for
709 male-biased orthologous genes in floral buds. The dioecious species
710 *Trichosanthes pilosa* represents the foreground (test) and other species represent
711 the background (reference).
712 Supplementary Table S10. Genes under intensified positive selection identified by
713 RELAX model in HyPhy and functions in NR, KEGG, Swissport and GO
714 databases for male-biased orthologous genes in floral buds. The dioecious species
715 *Trichosanthes pilosa* represents the foreground (test) and other species represent
716 the background (reference).
717 Supplementary Table S11. KEGG pathway enrichment analysis of female-biased
718 and male-biased genes in floral buds of the dioecious *Trichosanthes pilosa*.
719 Supplementary Table S12. Functions and references associated with abiotic stress
720 and immune responses, organ developments of male-biased genes under
721 significant positive selection ($P < 0.05$) in floral buds.
722 Supplementary Table S13. Functions and references associated with abiotic stress
723 and immune responses, organ developments of male-biased genes under
724 significant relaxed selection ($P < 0.05$) in floral buds.
725 Supplementary Table S14. The expressions and functions of some male-biased
726 genes associated with senescence, raceme inflorescence development and early

flowering in floral buds.

Data Availability

All RNA-Sequencing clean reads have been deposited in the databases of the National Center for Biotechnology Information (NCBI) under BioProject ID PRJNA899312.

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Figure Legends

Fig. 1 Floral buds and flowers at anthesis of females (A, B) and males (C) in *Trichosanthes pilosa.*

Fig. 2 Sex-biased gene expression for floral buds and flowers at anthesis in males and females of *Trichosanthes pilosa.* Volcano plots of average expression between female-biased, male-biased and unbiased genes in floral buds (A) and flowers at anthesis (B). M1 and F1 indicate male and female floral buds; M2 and F2 indicate male and female flowers at anthesis. The value of y coordinate represents $-\log_{10}$(FDR), and the value of x coordinate represents $\log_2$(Fold Change) identified by DESeq2. Heatmap of sex-biased gene expression (C) using hierarchical clustering analysis. Hierarchical gene clustering is based on Euclidean distance with an average of $\log_2$(FPKM) for differentially expressed genes. The color gradient represents from high to low (from red to green) gene expression.

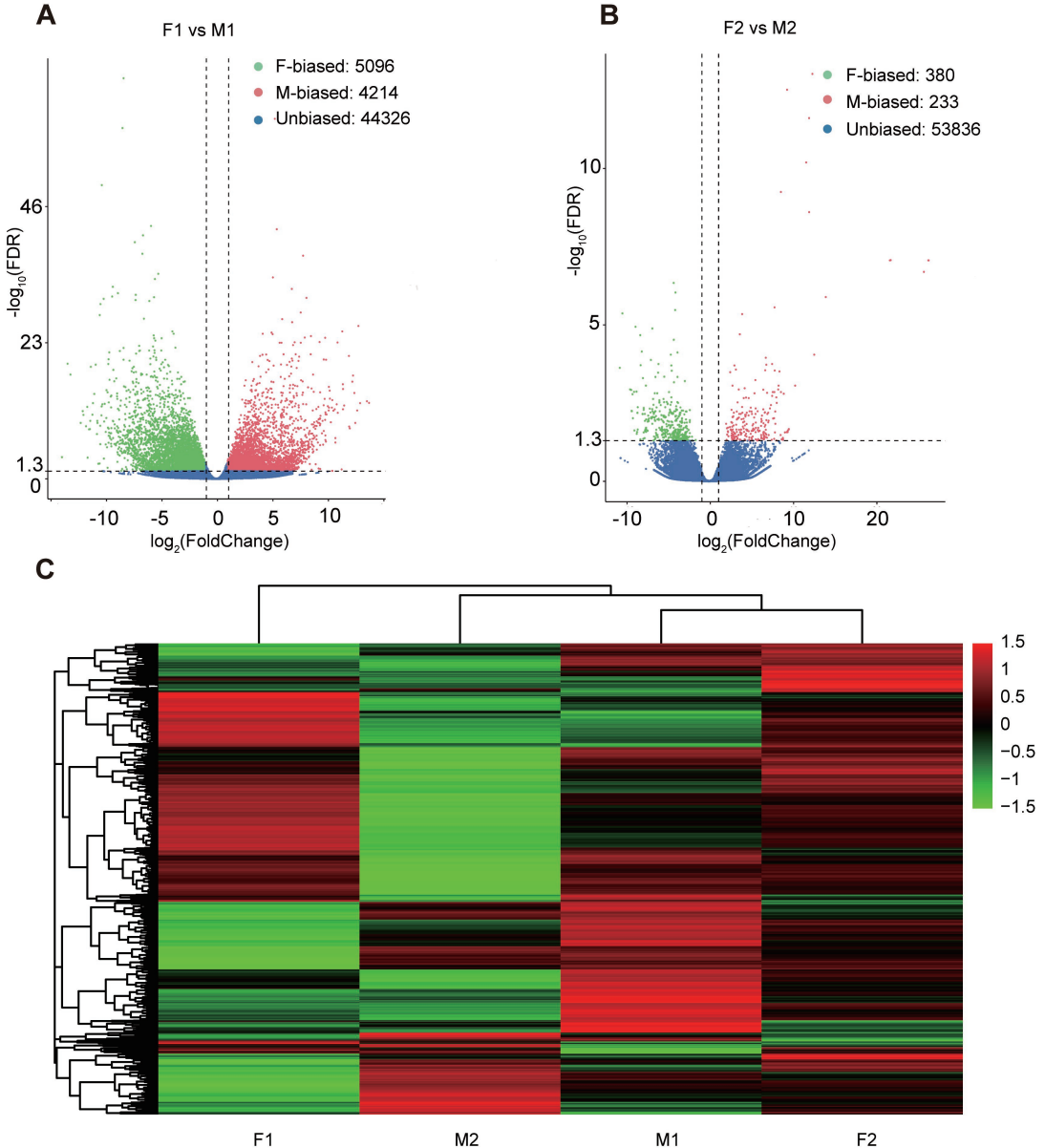
Fig. 3 The overlap between sex-biased and tissue-biased genes in two types of sexes

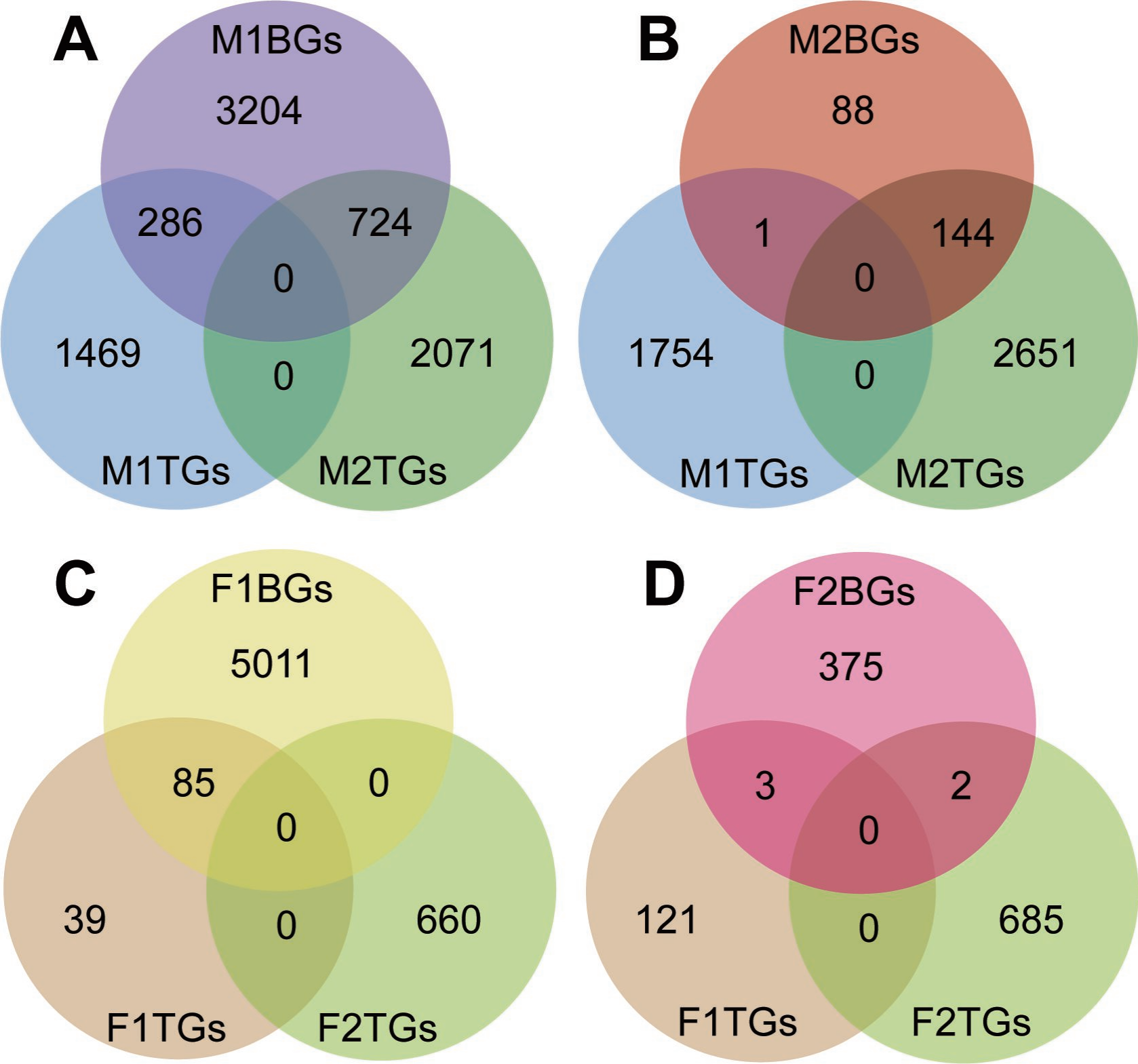
and tissues. Male-biased genes in floral buds (M1BGs) (A) or flowers at anthesis (M2BGs) (B) overlapped with tissue-biased genes of floral buds (M1TGs) and flowers at anthesis (M2TGs). Female-biased genes in floral buds (F1BGs) (C) or flowers at anthesis (F2BGs) (D) overlapped with tissue-biased genes of floral buds (F1TGs) and flowers at anthesis (F2TGs).

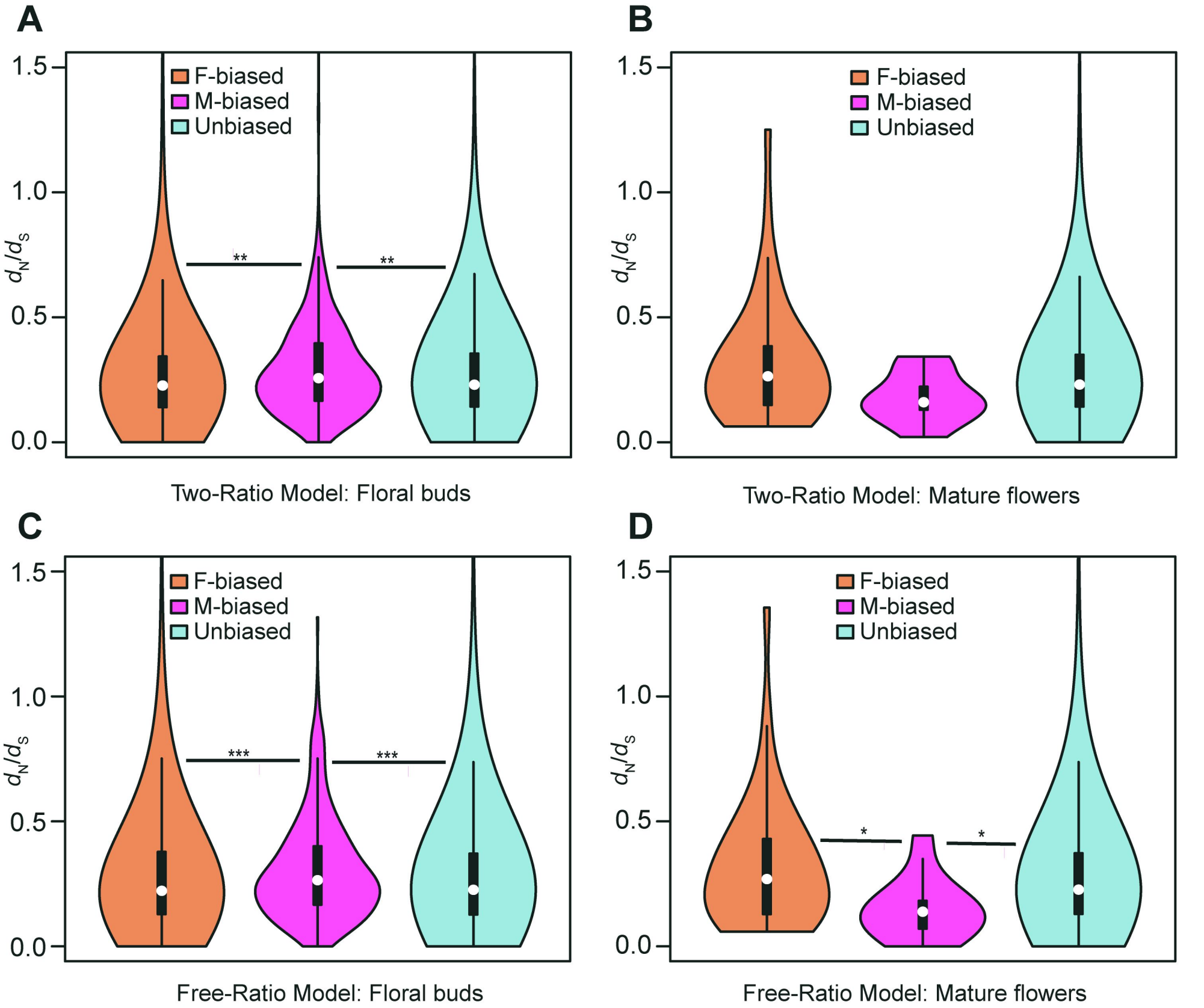
Fig. 4 Violin plots of d_N/d_S values ($0 < \omega < 2$) of female-biased, male-biased and unbiased genes in floral buds and flowers at anthesis of *Trichosanthes pilosa*. White dot indicates the median of d_N/d_S values for sex-biased and unbiased genes. Wilcoxon rank sum tests are used to test for significant differences (***$P < 0.0005$, **$P < 0.005$ and *$P < 0.05$). The distributions of d_N/d_S values for female-biased, male-biased and unbiased genes in floral buds (A) and flowers at anthesis (B) using 'two-ratio' branch model. The distributions of d_N/d_S values for female-biased, male-biased and unbiased genes in floral buds (C) and flowers at anthesis (D) using 'free-ratio' branch model.

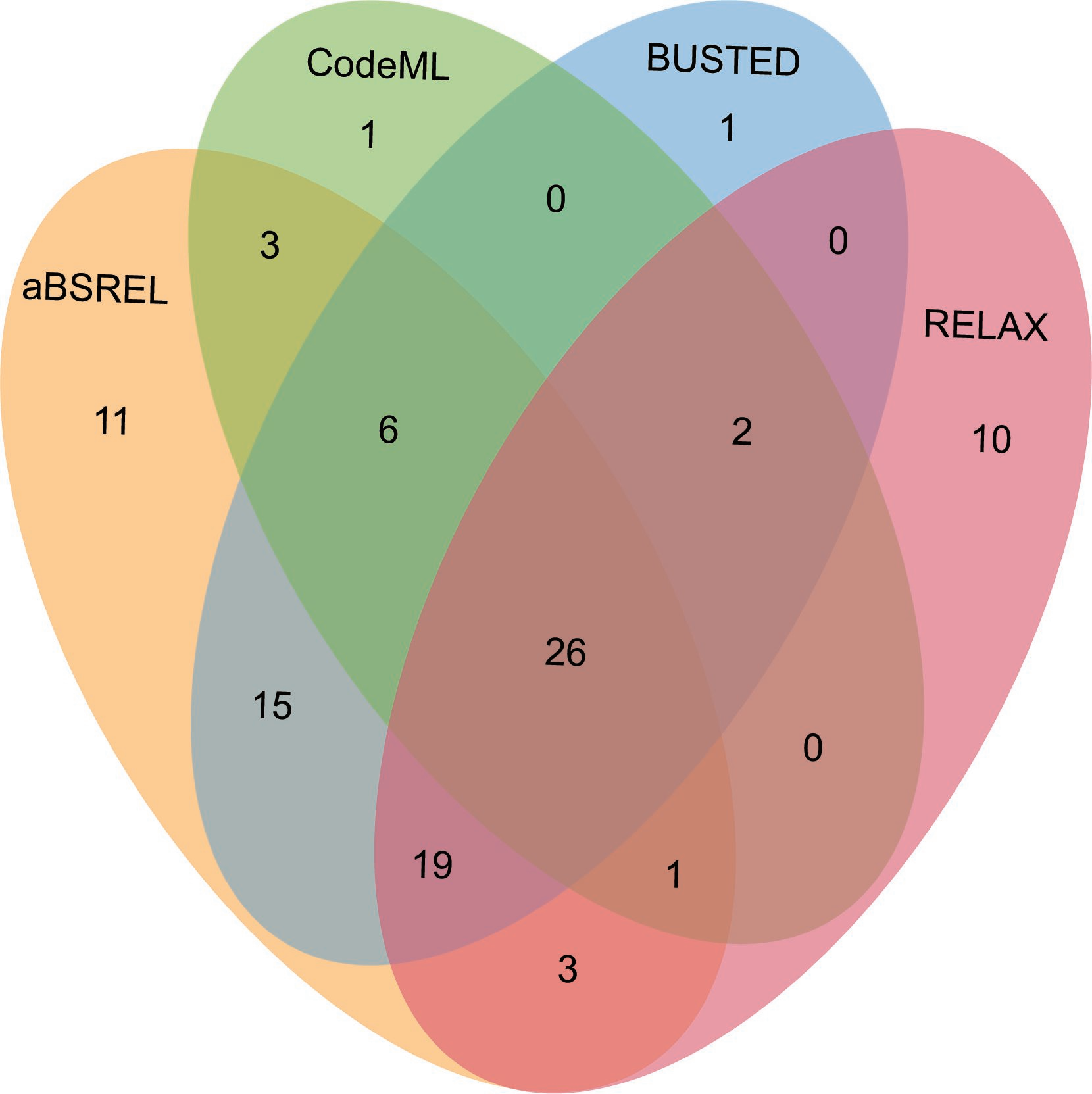
Fig. 5 Venn diagrams of male-biased genes detected to be under positive selection using aBSREL, BUSTED, CodeML and RELAX in floral buds.

A
B
C


A
F1 vs M1
F-biased: 5096
M-biased: 4214
Unbiased: 44326
-log10(FDR)
46
23
1.3
0
-10
-5
0
5
10
log2(FoldChange)
B
F2 vs M2
F-biased: 380
M-biased: 233
Unbiased: 53836
-log10(FDR)
10
5
1.3
0
-10
0
10
20
log2(FoldChange)
C
1.5
1
0.5
0
−0.5
−1
−1.5
F1
M2
M1
F2


A
M1BGs
3204
286
724
0
1469
0
2071
M1TGs
M2TGs
B
M2BGs
88
1
144
0
1754
0
2651
M1TGs
M2TGs
C
F1BGs
5011
85
0
0
39
0
660
F1TGs
F2TGs
D
F2BGs
375
3
2
0
121
0
685
F1TGs
F2TGs


A
B
C
D
F-biased
M-biased
Unbiased
d_N/d_S
1.5
1.0
0.5
0.0
**
***
*
Two-Ratio Model: Floral buds
Two-Ratio Model: Mature flowers
Free-Ratio Model: Floral buds
Free-Ratio Model: Mature flowers


CodeML
BUSTED
aBSREL
RELAX
1
1
0
3
0
11
6
2
10
26
15
0
19
1
3